CRYSTALLIZABLE JNK COMPLEXES

TECHNICAL FIELD OF INVENTION

The present invention relates to crystallizable complexes of a JNK protein, particularly JNK3, and adenosine monophosphate. The present invention also relates to a data storage medium encoded with the structural coordinates of crystallized molecules and molecular complexes which comprise the active site binding pockets of JNK3. A computer comprising such data storage material is capable of displaying such molecules and molecular complexes, or their structural homologues, as a graphical three-dimensional representation on a computer screen. This invention also relates to methods of using the structure coordinates to solve the structure of homologous proteins or protein complexes. In addition, this invention relates to methods of using the structure coordinates to screen for and design compounds, including inhibitory compounds, that bind to JNK3 or homologues thereof.

BACKGROUND OF THE INVENTION

Mammalian cells respond to extracellular stimuli by activating signaling cascades that are mediated by members of the mitogen-activated protein (MAP) kinase family, which include the extracellular signal regulated kinases (ERKs), the p38 MAP kinases and the c-Jun N-terminal kinases (JNKs). MAP kinases are serine/threonine kinases that are activated by dual phosphorylation of threonine and tyrosine at the Thr-X-Tyr segment in the activation loop. MAP kinases phosphorylate various substrates including transcription factors, which in turn regulate the expression of specific sets of genes and thus mediate a specific response to the stimulus.

Three distinct genes, Jnk1, Jnk2, Jnk3 have

been identified and at least ten different splicing isoforms of JNK exist in mammalian cells [S. Gupta et al., EMBO J., 15, pp. 2760-2770 (1996)]. Members of the JNK kinases are activated by proinflammatory cytokines tumor necrosis factor-alpha and interleukin-1 beta as well as environmental stress, such as anisomycin, UV irradiation, hypoxia, and osmotic shock [A. Minden et al., Biochemica et Biophysica Acta, 1333, F85-F104 (1997)]. Regulation & function of the JNK subgroup of MAP kinases. The down-stream substrates of JNKs include transcription factors c-Jun, ATF-2, Elk1, p53 and a cell death domain protein (DENN) [Y. Zhang et al. Proc. Natl. Acad. Sci. USA, 95, pp. 2586-2591 (1998)]. Each JNK isoform binds to these substrates with different affinities, suggesting a regulation of signaling pathways by substrate specificity of different JNKs *in vivo* (S. Gupta et al., 1996)

JNK1 and JNK2 are widely expressed in a variety of tissues. In contrast, JNK3 is selectively expressed in the brain and to a lesser extent in the heart and testis [S. Gupta et al., (1996); A. A. Mohit et al., Neuron, 14, pp. 67-78 (1995); J.H. Martin et al., Brain Res. Mol. Brain Res., 35, pp. 47-57 (1996)]. In the adult human brain, JNK3 expression is localized to a subpopulation of pyramidal neurons in the CA1, CA4 and subiculum regions of the hippocampus and layers 3 and 5 of the neocortex [A. A. Mohit et al. (1995)]. The CA1 neurons of patients with acute hypoxia showed strong nuclear JNK3-immunoreactivity compared to minimal, diffuse cytoplasmic staining of the hippocampal neurons from brain tissues of normal patients [Y. Zhang et al. (1998)]. In addition, JNK3 co-localizes immunochemically with neurons vulnerable in Alzheimer's disease [A. A. Mohit et al., (1995)]. Disruption of the JNK3 gene caused resistance of mice to the excitotoxic glutamate receptor agonist kainic acid, including the effects on seizure activity, AP-1 transcriptional activity and

apoptosis of hippocampal neurons, indicating that the JNK3 signaling pathway is a critical component in the pathogenesis of glutamate neurotoxicity (D. D. Yang et al., Nature, 389, pp. 865-870 (1997)]. Thus, selective modulation of JNK3 activity could potentially provide therapeutic intervention for neurodegenerative diseases such as stroke and epilepsy.

Despite the fact that the genes for various JNKs have been isolated and the amino acid sequences are known, no one has described X-ray crystal structural coordinate information of any of the JNKs. Such information would be extremely useful in identifying and designing potential inhibitors of various JNKs which, in turn, could have therapeutic utility.

SUMMARY OF THE INVENTION

Applicants have solved this problem by providing, for the first time, a crystallizable composition comprising unphosphorylated JNK3 in complex with MgAMP-PNP and the resulting crystal. The crystal was resolved at 2.3 Å resolution. Solving this crystal structure has allowed applicants to determine the key structural features of JNK3, particularly the shape of its substrate binding site.

The invention also provides a machine readable storage medium which comprises the structure coordinates of the JNK3 binding site. Such storage medium encoded with these data when read and utilized by a computer programmed with appropriate software displays, on a computer screen or similar viewing device, a three-dimensional graphical representation of a molecule or molecular complex comprising such binding sites or similarly shaped homologous binding pockets.

The invention also provides methods for designing, evaluating and identifying compounds which

bind to the aforementioned binding sites, as well as compounds produced by such methods. Such compounds are potential inhibitors of JNK3 or its homologues.

The invention also provides a method for determining at least a portion of the three-dimensional structure of molecules or molecular complexes which contain at least some structurally similar features to JNK3, particularly JNK1, JNK2 and other JNK isoforms. This is achieved by using at least some of the structural coordinates obtained for the unphosphorylated JNK3 in complex with MgAMP-PNP.

The invention also provides a method for crystallizing unphosphorylated JNK3 in complex with MgAMP-PNP.

BRIEF DESCRIPTION OF THE FIGURES

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Figure 1 lists the atomic structure coordinates for unphosphorylated JNK3 in complex with MgAMP-PNP as derived by X-ray diffraction from a crystal of that complex. The following abbreviations are used in Figure 1:

"Atom type" refers to the element whose coordinates are measured. The first letter in the column defines the element.

"X, Y, Z" crystallographically define the atomic position of the element measured.

"B" is a thermal factor that measures movement of the atom around its atomic center.

"Occ" is an occupancy factor that refers to the fraction of the molecules in which each atom occupies the position specified by the coordinates. A value of "1" indicates that each atom has the same conformation, i.e., the same position, in all molecules of the crystal.

Fig 1a is a structure-based sequence alignment of JNK3, ERK2, p38 and cAPK.

Fig 2a is a ribbon representation of the overall fold of JNK3 complexed with MgAMP-PNP.

Figure 2b is a stereoscopic view of the superimposed structures of JNK3/MgAMP-PNP and Erk2.

Fig 3 is stereoscopic view of the superimposed structures of JNK3 and cAPK.

Figure 4a is stereoscopic view of the active site of JNK3.

Fig 4b is a detailed comparison of the active site of JNK3 with that of cAPK.

Fig 5 is a substrate binding specificity of JNK isoforms.

Figure 6 shows a diagram of a system used to carry out the instructions encoded by the storage medium of Figures 7 and 8.

Figure 7 shows a cross section of a magnetic storage medium.

Figure 8 shows a cross section of a optically-readable data storage medium.

DETAILED DESCRIPTION OF THE INVENTION

The following abbreviations are used throughout the application:

A =	Ala =	Alanine	T =	Thr =	Threonine
V =	Val =	Valine	C =	Cys =	Cysteine
L =	Leu =	Leucine	Y =	Tyr =	Tyrosine
I =	Ile =	Isoleucine	N =	Asn =	Asparagine
P =	Pro =	Proline	Q =	Gln =	Glutamine
F =	Phe =	Phenylalanine	D =	Asp =	Aspartic Acid
W =	Trp =	Tryptophan	E =	Glu =	Glutamic Acid
M =	Met =	Methionine	K =	Lys =	Lysine
G =	Gly =	Glycine	R =	Arg =	Arginine
S =	Ser =	Serine	H =	His =	Histidine

Additional definitions are set forth in the specification where necessary.

In order that the invention described herein may be more fully understood, the following detailed description is set forth.

According to one embodiment, the invention provides a crystallizable composition comprising an unphosphorylated JNK protein complexed with adenosine monophosphate. The JNK protein in the crystallizable complexes of this invention, if it is JNK3 or a JNK3 variant (as opposed to JNK1 or JNK2), must be truncated at the N-terminus. Specifically, the JNK3 proteins contain an N-terminal extension of about 40 amino acids as compared to JNK1 and JNK2 proteins (see GenBank entries for JNK1, JNK2 and JNK3 proteins and their isoforms). Those 40 amino acid must be removed from JNK3 proteins in the crystallizable compositions of this invention.

In addition, any JNK protein in these crystallizable compositions preferably have a C-terminal truncation of about 20 amino acids. We have found that

the C-terminal truncation is necessary to obtain diffraction quality crystals.

The second component in these compositions is a non-hydrolyzable ATP analog or a suicidal substrate. Non-hydrolyzable ATP analogs useful in the crystallizable compositions of this invention include AMP-PCH_2P, AMP-PSP and AMP-PNP. An example of a suicidal substrate is FSBA. Preferably, the crystallizable compositions of this invention comprise AMP-PNP as the substrate. The third component is magnesium ions. Mg can be introduced by incubating the non-hydrolyzable ATP analog or suicide substrate with $MgCl_2$ prior to incubation with the JNK protein.

We have also determined that the buffer conditions of the composition are crucial for crystallization. Thus, the crystallizable compositions of this invention also comprise polyethylene glycol monomethyl ether at between about 10 to 30% v/v, ethylene glycol at between about 5 to 20% v/v, a reducing agent, such as ß-mercaptoethanol at between about 5 to 50 mM, and a buffer that maintains pH at between about 7.0 and 7.5. Preferably the buffer is 100 mM Hepes at pH 7.0.

The invention also relates to crystals of a JNK protein complexes with Mg and a non-hydrolyzable ATP analog or a suicidal substrate. These crystals are obtained from the above described compositions by standard crystallization protocols.

The invention also related to a method of making JNK-containing crystals. Such methods comprise the steps of:

a) obtaining a crystallizable composition comprising a JNK protein complexed with Mg and a non-hydrolyzable ATP analog or a suicidal substrate, as described above; and

b) subjecting said composition to conditions which promote crystallization.

In each of the above embodiments, it is preferred that the JNK protein be a JNK3, and in particular JNK3α1.

As mentioned above, applicants have solved the three-dimensional X-ray crystal structure of JNK3a1. The atomic coordinate data is presented in Figure 1.

In order to use the structure coordinates generated for the JNK3/MgAMP-PNP complex or one of its binding pockets or homologues thereof, it is often times necessary to convert them into a three-dimensional shape. This is achieved through the use of commercially available software that is capable of generating three-dimensional graphical representations of molecules or portions thereof from a set of structure coordinates.

Binding pockets, also referred to as binding sites in the present invention, are of significant utility in fields such as drug discovery. The association of natural ligands or substrates with the binding pockets of their corresponding receptors or enzymes is the basis of many biological mechanisms of action. Similarly, many drugs exert their biological effects through association with the binding pockets of receptors and enzymes. Such associations may occur with all or any parts of the binding pocket. An understanding of such associations will help lead to the design of drugs having more favorable associations with their target receptor or enzyme, and thus, improved biological effects. Therefore, this information is valuable in designing potential inhibitors of the binding sites of biologically important targets.

The term "binding pocket", as used herein, refers to a region of a molecule or molecular complex, that, as a result of its shape, favorably associates with another chemical entity or compound.

The term "JNK3-like binding pocket" refers to a portion of a molecule or molecular complex whose shape is sufficiently similar to the JNK3 binding pockets as to

bind common ligands. This commonality of shape is defined by a root mean square deviation from the structure coordinates of the backbone atoms of the amino acids that make up the binding pockets in JNK3 (as set forth in Figure 1) of not more than 1.5 A. The method of performing this calculation is described below.

The "active site binding pockets" or "active site" of JNK3 refers to the area on the JNK3 enzyme surface where the nucleotide substrate binds. In resolving the crystal structure of unphosphorylated JNK3α1 in complex with MgAMP-PNP, applicants have determined that JNK3 amino acids Ile70, Gly71, Ser72, Gly73, Ala74, Gln75, Gly76, Val78, Ala91, Lys93, Glu111, Ile124, Met146, Glu147, Leu148, Met149, Asp150, Ala151, Asn152, Gln155, Lys191, Ser193, Asn194, Val196 and Leu206 are within 5 Å of and therefore close enough to interact with MgAMP-PNP. Thus, a binding pocket defined by the structural coordinates of those amino acids, as set forth in Figure 1; or a binding pocket whose root mean square deviation from the structure coordinates of the backbone atoms of those amino acids of not more than 1.5 Å is considered a JNK3-like binding pocket of this invention.

Applicants have also determined that in addition to the JNK3 amino acids set forth above, Ile77, Cys79, Ala80, Val90, Ile92, Lys94, Leu95, His104, Arg107, Ser125, Leu144, Val145, Leu153, Cys154, Asp189, Pro192, Ile195, Val197, Lys204 and Asp207 are within 8 Å of bound MgAMP-PNP and therefore are also close enough to interact with that substrate. Thus, in a preferred embodiment, a binding pocket defined by the structural coordinates of the amino acids within 8 Å bound MgAMP-PNP, as set forth in Figure 1; or a binding pocket whose root mean square deviation from the structure coordinates of the backbone atoms of those amino acids of not more than 1.5 Å is

considered a preferred JNK3-like binding pocket of this invention.

It will be readily apparent to those of skill in the art that the numbering of amino acids in other isoforms of JNK may be different than that set forth for JNK3α1. Corresponding amino acids in other isoforms of JNK are easily identified by visual inspection of the amino acid sequences or by using commercially available homology software programs.

Each of those amino acids of JNK3α1 is defined by a set of structure coordinates set forth in Figure 1. The term "structure coordinates" refers to Cartesian coordinates derived from mathematical equations related to the patterns obtained on diffraction of a monochromatic beam of X-rays by the atoms (scattering centers) of a protein or protein-ligand complex in crystal form. The diffraction data are used to calculate an electron density map of the repeating unit of the crystal. The electron density maps are then used to establish the positions of the individual atoms of the enzyme or enzyme complex.

Those of skill in the art understand that a set of structure coordinates for an enzyme or an enzyme-complex or a portion thereof, is a relative set of points that define a shape in three dimensions. Thus, it is possible that an entirely different set of coordinates could define a similar or identical shape. Moreover, slight variations in the individual coordinates will have little effect on overall shape. In terms of binding pockets, these variations would not be expected to significantly alter the nature of ligands that could associate with those pockets.

The term "associating with" refers to a condition of proximity between a chemical entity or compound, or portions thereof, and a binding pocket or binding site on a protein. The association may be non-covalent -- wherein the juxtaposition is energetically

favored by hydrogen bonding or van der Waals or electrostatic interactions -- or it may be covalent.

The variations in coordinates discussed above may be generated because of mathematical manipulations of the JNK3/MgAMP-PNP structure coordinates. For example, the structure coordinates set forth in Figure 1 could be manipulated by crystallographic permutations of the structure coordinates, fractionalization of the structure coordinates, integer additions or subtractions to sets of the structure coordinates, inversion of the structure coordinates or any combination of the above.

Alternatively, modifications in the crystal structure due to mutations, additions, substitutions, and/or deletions of amino acids, or other changes in any of the components that make up the crystal could also account for variations in structure coordinates. If such variations are within an acceptable standard error as compared to the original coordinates, the resulting three-dimensional shape is considered to be the same. Thus, for example, a ligand that bound to the active site binding pocket of JNK3 would also be expected to bind to another binding pocket whose structure coordinates defined a shape that fell within the acceptable error.

Various computational analyses are therefore necessary to determine whether a molecule or the binding pocket portion thereof is sufficiently similar to the JNK3 binding pockets described above. Such analyses may be carried out in well known software applications, such as the Molecular Similarity application of QUANTA (Molecular Simulations Inc., San Diego, CA) version 4.1, and as described in the accompanying User's Guide.

The Molecular Similarity application permits comparisons between different structures, different conformations of the same structure, and different parts of the same structure. The procedure used in Molecular Similarity to compare structures is divided into four

steps: 1) load the structures to be compared; 2) define the atom equivalences in these structures; 3) perform a fitting operation; and 4) analyze the results.

Each structure is identified by a name. One structure is identified as the target (i.e., the fixed structure); all remaining structures are working structures (i.e., moving structures). Since atom equivalency within QUANTA is defined by user input, for the purpose of this invention we will define equivalent atoms as protein backbone atoms (N, Cα, C and O) for all conserved residues between the two structures being compared. We will also consider only rigid fitting operations.

When a rigid fitting method is used, the working structure is translated and rotated to obtain an optimum fit with the target structure. The fitting operation uses an algorithm that computes the optimum translation and rotation to be applied to the moving structure, such that the root mean square difference of the fit over the specified pairs of equivalent atom is an absolute minimum. This number, given in angstroms, is reported by QUANTA.

For the purpose of this invention, any molecule or molecular complex or binding pocket thereof that has a root mean square deviation of conserved residue backbone atoms (N, Cα, C, O) of less than 1.5 Å when superimposed on the relevant backbone atoms described by structure coordinates listed in Figure 1 are considered identical. More preferably, the root mean square deviation is less than 1.0 Å.

The term "root mean square deviation" means the square root of the arithmetic mean of the squares of the deviations from the mean. It is a way to express the deviation or variation from a trend or object. For purposes of this invention, the "root mean square deviation" defines the variation in the backbone of a

protein from the backbone of JNK3 or a binding pocket portion thereof, as defined by the structure coordinates of JNK3 described herein.

Therefore, according to another embodiment of this invention is provided a machine-readable data storage medium, comprising a data storage material encoded with machine readable data which, when used by a machine programmed with instructions for using said data, displays a graphical three-dimensional representation of a molecule or molecular complex comprising a binding pocket defined by structure coordinates of JNK3 amino acids Ile70, Gly71, Ser72, Gly73, Ala74, Gln75, Gly76, Val78, Ala91, Lys93, Glu111, Ile124, Met146, Glu147, Leu148, Met149, Asp150, Ala151, Asn152, Gln155, Lys191, Ser193, Asn194, Val196 and Leu206 according to Figure 1, or a homologue of said molecule or molecular complex, wherein said homologue comprises a binding pocket that has a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5 Å.

Preferably, the machine readable data, when used by a machine programmed with instructions for using said data, displays a graphical three-dimensional representation of a molecule or molecular complex comprising a binding pocket defined by structure coordinates of JNK3 amino acids Ile70, Gly71, Ser72, Gly73, Ala74, Gln75, Gly76, Ile77, Val78, Cys79, Ala80, Val90, Ala91, Ile92, Lys93, Lys94, Leu95, His104, Arg107, Glu111, Ile124, Ser125, Leu144, Val145, Met146, Glu147, Leu148, Met149, Asp150, Ala151, Asn152, Leu153, Cys154, Gln155, Asp189, Lys191, Pro192, Ser193, Asn194, Ile195, Val196 Val197, Lys204, Leu206 and Asp207 according to Figure 1, or a homologue of said molecule or molecular complex, wherein said homologue comprises a binding pocket that has a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5 Å.

Even more preferred is a machine-readable data storage medium that is capable of displaying a graphical three-dimensional representation of a molecule or molecular complex that is defined by the structure coordinates of all of the amino acids in Figure 1 or a homologue of said molecule or molecular complex, wherein said homologue has a root mean square deviation from the backbone atoms of all of the amino acids in Figure 1 of not more than 1.5 Å.

According to an alternate embodiment, the machine-readable data storage medium comprises a data storage material encoded with a first set of machine readable data which comprises the Fourier transform of the structure coordinates set forth in Figure 1, and which, when using a machine programmed with instructions for using said data, can be combined with a second set of machine readable data comprising the X-ray diffraction pattern of a molecule or molecular complex to determine at least a portion of the structure coordinates corresponding to the second set of machine readable data.

For example, the Fourier transform of the structure coordinates set forth in Figure 1 may be used to determine at least a portion of the structure coordinates of other JNKs, such as JNK1, JNK2 and isoforms of JNK1, JNK2 or JNK3.

Figure 6 demonstrates one version of these embodiments. System 10 includes a computer 11 comprising a central processing unit ("CPU") 20, a working memory 22 which may be, e.g., RAM (random-access memory) or "core" memory, mass storage memory 24 (such as one or more disk drives or CD-ROM drives), one or more cathode-ray tube ("CRT") display terminals 26, one or more keyboards 28, one or more input lines 30, and one or more output lines 40, all of which are interconnected by a conventional bi-directional system bus 50.

Input hardware 36, coupled to computer 11 by input lines 30, may be implemented in a variety of ways.

Machine-readable data of this invention may be inputted via the use of a modem or modems 32 connected by a telephone line or dedicated data line 34. Alternatively or additionally, the input hardware 36 may comprise CD-ROM drives or disk drives 24. In conjunction with display terminal 26, keyboard 28 may also be used as an input device.

Output hardware 46, coupled to computer 11 by output lines 40, may similarly be implemented by conventional devices. By way of example, output hardware 46 may include CRT display terminal 26 for displaying a graphical representation of a binding pocket of this invention using a program such as QUANTA as described herein. Output hardware might also include a printer 42, so that hard copy output may be produced, or a disk drive 24, to store system output for later use.

In operation, CPU 20 coordinates the use of the various input and output devices 36, 46, coordinates data accesses from mass storage 24 and accesses to and from working memory 22, and determines the sequence of data processing steps. A number of programs may be used to process the machine-readable data of this invention. Such programs are discussed in reference to the computational methods of drug discovery as described herein. Specific references to components of the hardware system 10 are included as appropriate throughout the following description of the data storage medium.

According to an alternate embodiment, the present invention provides a computer for producing a three-dimensional representation of a molecule or molecular complex, wherein said molecule or molecular complex comprises a binding pocket defined by Ile70, Gly71, Ser72, Gly73, Ala74, Gln75, Gly76, Val78, Ala91, Lys93, Glu111, Ile124, Met146, Glu147, Leu148, Met149, Asp150, Ala151, Asn152, Gln155, Lys191, Ser193, Asn194, Val196 and Leu206 according to Figure 1, or a homologue

of said molecule or molecular complex, wherein said homologue comprises a binding pocket that has a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5 Å, wherein said computer comprises:

(a) a machine readable data storage medium comprising a data storage material encoded with machine-readable data, wherein said machine readable data comprises the structure coordinates of JNK3 or portions thereof;

(b) a working memory for storing instructions for processing said machine-readable data;

(c) a central-processing unit coupled to said working memory and to said machine-readable data storage medium, for processing said machine-readable data into said three-dimensional representation; and

(d) an output hardware coupled to said central processing unit, for receiving said three Dimensional representation.

Preferably, the computer produces a three-dimensional representation of a molecule or molecular complex, wherein said molecule or molecular complex comprises a binding pocket defined by the binding pocket is defined by structure coordinates of JNK3 amino acids Ile70, Gly71, Ser72, Gly73, Ala74, Gln75, Gly76, Ile77, Val78, Cys79, Ala80, Val90, Ala91, Ile92, Lys93, Lys94, Leu95, His104, Arg107, Glu111, Ile124, Ser125, Leu144, Val145, Met146, Glu147, Leu148, Met149, Asp150, Ala151, Asn152, Leu153, Cys154, Gln155, Asp189, Lys191, Pro192, Ser193, Asn194, Ile195, Val196 Val197, Lys204, Leu206 and Asp207 according to Figure 1, or a homologue of said molecule or molecular complex, wherein said homologue comprises a binding pocket that has a root mean square

deviation from the backbone atoms of said amino acids of not more than 1.5 Å.

Figure 7 shows a cross section of a magnetic data storage medium 100 which can be encoded with a machine-readable data that can be carried out by a system such as system 10 of Figure 6. Medium 100 can be a conventional floppy diskette or hard disk, having a suitable substrate 101, which may be conventional, and a suitable coating 102, which may be conventional, on one or both sides, containing magnetic domains (not visible) whose polarity or orientation can be altered magnetically. Medium 100 may also have an opening (not shown) for receiving the spindle of a disk drive or other data storage device 24.

The magnetic domains of coating 102 of medium 100 are polarized or oriented so as to encode in manner which may be conventional, machine readable data such as that described herein, for execution by a system such as system 10 of Figure 6.

Figure 8 shows a cross section of an optically-readable data storage medium 110 which also can be encoded with such a machine-readable data, or set of instructions, which can be carried out by a system such as system 10 of Figure 6. Medium 110 can be a conventional compact disk read only memory (CD-ROM) or a rewritable medium such as a magneto-optical disk which is optically readable and magneto-optically writable. Medium 100 preferably has a suitable substrate 111, which may be conventional, and a suitable coating 112, which may be conventional, usually of one side of substrate 111.

In the case of CD-ROM, as is well known, coating 112 is reflective and is impressed with a plurality of pits 113 to encode the machine-readable data. The arrangement of pits is read by reflecting laser light off the surface of coating 112. A protective

coating 114, which preferably is substantially transparent, is provided on top of coating 112.

In the case of a magneto-optical disk, as is well known, coating 112 has no pits 113, but has a plurality of magnetic domains whose polarity or orientation can be changed magnetically when heated above a certain temperature, as by a laser (not shown). The orientation of the domains can be read by measuring the polarization of laser light reflected from coating 112. The arrangement of the domains encodes the data as described above.

Thus, in accordance with the present invention, data capable of displaying the three dimensional structure of JNK3 and portions thereof and their structurally similar homologues is stored in a machine-readable storage medium, which is capable of displaying a graphical three-dimensional representation of the structure.

The JNK3 X-ray coordinate data, when used in conjunction with a computer programmed with software to translate those coordinates into the 3-dimensional structure of JNK3 may be used for a variety of purposes, such as drug discovery.

For example, the structure encoded by the data may be computationally evaluated for its ability to associate with chemical entities. Chemical entities that associate with JNK3 may inhibit JNK3, and are potential drug candidates. Alternatively, the structure encoded by the data may be displayed in a graphical three-dimensional representation on a computer screen. This allows visual inspection of the structure, as well as visual inspection of the structure's association with chemical entities.

Thus, according to another embodiment, the invention relates to a method for evaluating the potential of a chemical entity to associate with a molecule or molecular complex comprising a binding pocket

defined by structure coordinates of JNK3 amino acids Ile70, Gly71, Ser72, Gly73, Ala74, Gln75, Gly76, Val78, Ala91, Lys93, Glu111, Ile124, Met146, Glu147, Leu148, Met149, Asp150, Ala151, Asn152, Gln155, Lys191, Ser193, Asn194, Val196 and Leu206 according to Figure 1, or a homologue of said molecule or molecular complex, wherein said homologue comprises a binding pocket that has a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5 Å.

This method comprises the steps of: a) employing computational means to perform a fitting operation between the chemical entity and a binding pocket of the molecule or molecular complex; b) analyzing the results of said fitting operation to quantify the association between the chemical entity and the binding pocket; and c) outputting said quantified association to a suitable output hardware, such as a CRT display terminal, a printer or a disk drive, as described previously. The term "chemical entity", as used herein, refers to chemical compounds, complexes of at least two chemical compounds, and fragments of such compounds or complexes.

Preferably, the method evaluates the potential of a chemical entity to associate with a molecule or molecular complex comprising a binding pocket defined by structure coordinates of JNK3 amino acids Ile70, Gly71, Ser72, Gly73, Ala74, Gln75, Gly76, Ile77, Val78, Cys79, Ala80, Val90, Ala91, Ile92, Lys93, Lys94, Leu95, His104, Arg107, Glu111, Ile124, Ser125, Leu144, Val145, Met146, Glu147, Leu148, Met149, Asp150, Ala151, Asn152, Leu153, Cys154, Gln155, Asp189, Lys191, Pro192, Ser193, Asn194, Ile195, Val196 Val197, Lys204, Leu206 and Asp207 according to Figure 1, or a homologue of said molecule or molecular complex, wherein said homologue comprises a binding pocket that has a root mean square deviation from the backbone atoms of said amino acids of not more than

1.5 Å.

Even more preferably, the method evaluates the potential of a chemical entity to associate with a molecule or molecular complex defined by structure coordinates of all of the JNK3 amino acids, as set forth in Figure 1, or a homologue of said molecule or molecular complex having a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5 Å.

Alternatively, the structural coordinates of the JNK3 binding pocket can be utilized in a method for identifying a potential agonist or antagonist of a molecule comprising a JNK3-like binding pocket. This method comprises the steps of:

a. using the atomic coordinates of Ile70, Gly71, Ser72, Gly73, Ala74, Gln75, Gly76, Val78, Ala91, Lys93, Glu111, Ile124, Met146, Glu147, Leu148, Met149, Asp150, Ala151, Asn152, Gln155, Lys191, Ser193, Asn194, Val196 and Leu206 according to Figure 1 ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5 Å, to generate a three-dimensional structure of molecule comprising a JNK3-like binding pocket;

b. employing said three-dimensional structure to design or select said potential agonist or antagonist;

c. synthesizing said agonist or antagonist; and

d. contacting said agonist or antagonist with said molecule to determine the ability of said potential agonist or antagonist to interact with said molecule.

More preferred is when the atomic coordinates of Ile70, Gly71, Ser72, Gly73, Ala74, Gln75, Gly76, Ile77, Val78, Cys79, Ala80, Val90, Ala91, Ile92, Lys93, Lys94, Leu95, His104, Arg107, Glu111, Ile124, Ser125, Leu144, Val145, Met146, Glu147, Leu148, Met149, Asp150, Ala151, Asn152, Leu153, Cys154, Gln155, Asp189, Lys191,

Pro192, Ser193, Asn194, Ile195, Val196 Val197, Lys204, Leu206 and Asp207 according to Figure 1 ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5 Å, are used to generate a three-dimensional structure of molecule comprising a JNK3-like binding pocket.

Most preferred is when the atomic coordinates of all the amino acids of JNK3 according to Figure 1 ± a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5 Å, are used to generate a three-dimensional structure of molecule comprising a JNK3-like binding pocket.

For the first time, the present invention permits the use of molecular design techniques to identify, select and design chemical entities, including inhibitory compounds, capable of binding to JNK3-like binding pockets.

Applicants' elucidation of binding sites on JNK3 provides the necessary information for designing new chemical entities and compounds that may interact with JNK3-like binding pockets, in whole or in part.

Throughout this section, discussions about the ability of an entity to bind to, associate with or inhibit a JNK3-like binding pocket refers to features of the entity alone. Assays to determine if a compound binds to JNK3 are well known in the art and are exemplified below.

The design of compounds that bind to or inhibit JNK3-like binding pockets according to this invention generally involves consideration of two factors. First, the entity must be capable of physically and structurally associating with parts or all of the JNK3-like binding pockets. Non-covalent molecular interactions important in this association include hydrogen bonding, van der Waals interactions, hydrophobic interactions and electrostatic interactions.

Second, the entity must be able to assume a conformation that allows it to associate with the JNK3-like binding pocket directly. Although certain portions of the entity will not directly participate in these associations, those portions of the entity may still influence the overall conformation of the molecule. This, in turn, may have a significant impact on potency. Such conformational requirements include the overall three-dimensional structure and orientation of the chemical entity in relation to all or a portion of the binding pocket, or the spacing between functional groups of an entity comprising several chemical entities that directly interact with the JNK3-like binding pocket or homologues thereof.

The potential inhibitory or binding effect of a chemical entity on a JNK3-like binding pocket may be analyzed prior to its actual synthesis and testing by the use of computer modeling techniques. If the theoretical structure of the given entity suggests insufficient interaction and association between it and the JNK3-like binding pocket, testing of the entity is obviated. However, if computer modeling indicates a strong interaction, the molecule may then be synthesized and tested for its ability to bind to a JNK3-like binding pocket. This may be achieved by testing the ability of the molecule to inhibit JNK3 using the assays described in Example 6. In this manner, synthesis of inoperative compounds may be avoided.

A potential inhibitor of a JNK3-like binding pocket may be computationally evaluated by means of a series of steps in which chemical entities or fragments are screened and selected for their ability to associate with the JNK3-like binding pockets.

One skilled in the art may use one of several methods to screen chemical entities or fragments for their ability to associate with a JNK3-like binding pocket. This process may begin by visual inspection of,

for example, a JNK3-like binding pocket on the computer screen based on the JNK3 structure coordinates in Figure 1 or other coordinates which define a similar shape generated from the machine-readable storage medium. Selected fragments or chemical entities may then be positioned in a variety of orientations, or docked, within that binding pocket as defined supra. Docking may be accomplished using software such as Quanta and Sybyl, followed by energy minimization and molecular dynamics with standard molecular mechanics force fields, such as CHARMM and AMBER.

Specialized computer programs may also assist in the process of selecting fragments or chemical entities. These include:

1. GRID (P. J. Goodford, "A Computational Procedure for Determining Energetically Favorable Binding Sites on Biologically Important Macromolecules", J. Med. Chem., 28, pp. 849-857 (1985)). GRID is available from Oxford University, Oxford, UK.

2. MCSS (A. Miranker et al., "Functionality Maps of Binding Sites: A Multiple Copy Simultaneous Search Method." Proteins: Structure, Function and Genetics, 11, pp. 29-34 (1991)). MCSS is available from Molecular Simulations, San Diego, CA.

3. AUTODOCK (D. S. Goodsell et al., "Automated Docking of Substrates to Proteins by Simulated Annealing", Proteins: Structure, Function, and Genetics, 8, pp. 195-202 (1990)). AUTODOCK is available from Scripps Research Institute, La Jolla, CA.

4. DOCK (I. D. Kuntz et al., "A Geometric Approach to Macromolecule-Ligand Interactions", J. Mol. Biol., 161, pp. 269-288 (1982)). DOCK is available from University of California, San Francisco, CA.

Once suitable chemical entities or fragments have been selected, they can be assembled into a single compound or complex. Assembly may be preceded by visual inspection of the relationship of the fragments to each other on the three-dimensional image displayed on a computer screen in relation to the structure coordinates of JNK3. This would be followed by manual model building using software such as Quanta or Sybyl [Tripos Associates, St. Louis, MO].

Useful programs to aid one of skill in the art in connecting the individual chemical entities or fragments include:

1. CAVEAT (P. A. Bartlett et al, "CAVEAT: A Program to Facilitate the Structure-Derived Design of Biologically Active Molecules", in Molecular Recognition in Chemical and Biological Problems", Special Pub., Royal Chem. Soc., 78, pp. 182-196 (1989); G. Lauri and P. A. Bartlett, "CAVEAT: a Program to Facilitate the Design of Organic Molecules", J. Comput. Aided Mol. Des. , 8, pp. 51-66 (1994)). CAVEAT is available from the University of California, Berkeley, CA.

2. 3D Database systems such as ISIS (MDL Information Systems, San Leandro, CA). This area is reviewed in Y. C. Martin, "3D Database Searching in Drug Design", J. Med. Chem., 35, pp. 2145-2154 (1992).

3. HOOK (M. B. Eisen et al, "HOOK: A Program for Finding Novel Molecular Architectures that Satisfy the Chemical and Steric Requirements of a Macromolecule Binding Site", Proteins: Struct., Funct., Genet., 19, pp. 199-221 (1994). HOOK is available from Molecular Simulations, San Diego, CA.

Instead of proceeding to build an inhibitor of a JNK3-like binding pocket in a step-wise fashion one fragment or chemical entity at a time as described above,

inhibitory or other JNK3 binding compounds may be designed as a whole or "de novo" using either an empty binding site or optionally including some portion(s) of a known inhibitor(s). There are many de novo ligand design methods including:

1. LUDI (H.-J. Bohm, "The Computer Program LUDI: A New Method for the De Novo Design of Enzyme Inhibitors", J. Comp. Aid. Molec. Design, 6, pp. 61-78 (1992)). LUDI is available from Molecular Simulations Incorporated, San Diego, CA.

2. LEGEND (Y. Nishibata et al., Tetrahedron, 47, p. 8985 (1991)). LEGEND is available from Molecular Simulations Incorporated, San Diego, CA.

3. LeapFrog (available from Tripos Associates, St. Louis, MO).

4. SPROUT (V. Gillet et al, "SPROUT: A Program for Structure Generation)", J. Comput. Aided Mol. Design, 7, pp. 127-153 (1993)). SPROUT is available from the University of Leeds, UK.

Other molecular modeling techniques may also be employed in accordance with this invention [see, e.g., N. C. Cohen et al., "Molecular Modeling Software and Methods for Medicinal Chemistry, J. Med. Chem., 33, pp. 883-894 (1990); see also, M. A. Navia and M. A. Murcko, "The Use of Structural Information in Drug Design", Current Opinions in Structural Biology, 2, pp. 202-210 (1992); L. M. Balbes et al., "A Perspective of Modern Methods in Computer-Aided Drug Design", in Reviews in Computational Chemistry, Vol. 5, K. B. Lipkowitz and D. B. Boyd, Eds., VCH, New York, pp. 337-380 (1994); see also, W. C. Guida, "Software For Structure-Based Drug Design", Curr. Opin. Struct. Biology,, 4, pp. 777-781 (1994)].

Once a compound has been designed or selected by the above methods, the efficiency with which that entity may bind to an JNK3 binding pocket may be tested and optimized by computational evaluation. For example, an effective JNK3 binding pocket inhibitor must preferably demonstrate a relatively small difference in energy between its bound and free states (i.e., a small deformation energy of binding). Thus, the most efficient JNK3 binding pocket inhibitors should preferably be designed with a deformation energy of binding of not greater than about 10 kcal/mole, more preferably, not greater than 7 kcal/mole. JNK3 binding pocket inhibitors may interact with the binding pocket in more than one conformation that is similar in overall binding energy. In those cases, the deformation energy of binding is taken to be the difference between the energy of the free entity and the average energy of the conformations observed when the inhibitor binds to the protein.

An entity designed or selected as binding to an JNK3 binding pocket may be further computationally optimized so that in its bound state it would preferably lack repulsive electrostatic interaction with the target enzyme and with the surrounding water molecules. Such non-complementary electrostatic interactions include repulsive charge-charge, dipole-dipole and charge-dipole interactions.

Specific computer software is available in the art to evaluate compound deformation energy and electrostatic interactions. Examples of programs designed for such uses include: Gaussian 94, revision C (M. J. Frisch, Gaussian, Inc., Pittsburgh, PA ©1995); AMBER, version 4.1 (P. A. Kollman, University of California at San Francisco, ©1995); QUANTA/CHARMM (Molecular Simulations, Inc., San Diego, CA ©1995); Insight II/Discover (Molecular Simulations, Inc., San Diego, CA ©1995); DelPhi (Molecular Simulations, Inc., San Diego, CA ©1995); and AMSOL (Quantum Chemistry

Program Exchange, Indiana University). These programs may be implemented, for instance, using a Silicon Graphics workstation such as an Indigo2 with "IMPACT" graphics. Other hardware systems and software packages will be known to those skilled in the art.

Another approach enabled by this invention, is the computational screening of small molecule databases for chemical entities or compounds that can bind in whole, or in part, to a JNK3 binding pocket. In this screening, the quality of fit of such entities to the binding site may be judged either by shape complementarity or by estimated interaction energy [E. C. Meng et al., J. Comp. Chem., 13, pp. 505-524 (1992)].

According to another embodiment, the invention provides compounds which associate with a JNK3-like binding pocket produced or identified by the method set forth above.

The structure coordinates set forth in Figure 1 can also be used to aid in obtaining structural information about another crystallized molecule or molecular complex. This may be achieved by any of a number of well-known techniques, including molecular replacement.

Therefore, in another embodiment this invention provides a method of utilizing molecular replacement to obtain structural information about a molecule or molecular complex whose structure is unknown comprising the steps of:

a) crystallizing said molecule or molecular complex of unknown structure;

b) generating an X-ray diffraction pattern from said crystallized molecule or molecular complex; and

c) applying at least a portion of the structure coordinates set forth in Figure 1 to the X-ray diffraction pattern to generate a three-dimensional

electron density map of the molecule or molecular complex whose structure is unknown.

By using molecular replacement, all or part of the structure coordinates of the JNK3/MgAMP-PNP complex as provided by this invention (and set forth in Figure 1) can be used to determine the structure of a crystallized molecule or molecular complex whose structure is unknown more quickly and efficiently than attempting to determine such information ab initio.

Molecular replacement provides an accurate estimation of the phases for an unknown structure. Phases are a factor in equations used to solve crystal structures that can not be determined directly. Obtaining accurate values for the phases, by methods other than molecular replacement, is a time-consuming process that involves iterative cycles of approximations and refinements and greatly hinders the solution of crystal structures. However, when the crystal structure of a protein containing at least a homologous portion has been solved, the phases from the known structure provide a satisfactory estimate of the phases for the unknown structure.

Thus, this method involves generating a preliminary model of a molecule or molecular complex whose structure coordinates are unknown, by orienting and positioning the relevant portion of the JNK3/MgAMP-PNP complex according to Figure 1 within the unit cell of the crystal of the unknown molecule or molecular complex so as best to account for the observed X-ray diffraction pattern of the crystal of the molecule or molecular complex whose structure is unknown. Phases can then be calculated from this model and combined with the observed X-ray diffraction pattern amplitudes to generate an electron density map of the structure whose coordinates are unknown. This, in turn, can be subjected to any well-known model building and structure refinement techniques to provide a final, accurate structure of the

unknown crystallized molecule or molecular complex [E. Lattman, "Use of the Rotation and Translation Functions", in Meth. Enzymol., 115, pp. 55-77 (1985); M. G. Rossmann, ed., "The Molecular Replacement Method", Int. Sci. Rev. Ser., No. 13, Gordon & Breach, New York (1972)].

The structure of any portion of any crystallized molecule or molecular complex that is sufficiently homologous to any portion of the JNK3/MgAMP-PNP complex can be resolved by this method.

In a preferred embodiment, the method of molecular replacement is utilized to obtain structural information about another JNK, such as JNK1, JNK2, or isoforms of JNK1, JNK2 or JNK3. The structure coordinates of JNK3 as provided by this invention are particularly useful in solving the structure of other isoforms of JNK3 or JNK3 complexes.

Furthermore, the structure coordinates of JNK3 as provided by this invention are useful in solving the structure of JNK3 proteins that have amino acid substitutions, additions and/or deletions (referred to collectively as "JNK3 mutants", as compared to naturally occurring JNK3 isoforms. These JNK3 mutants may optionally be crystallized in co-complex with a chemical entity, such as a non-hydrolyzable ATP analogue or a suicide substrate. The crystal structures of a series of such complexes may then be solved by molecular replacement and compared with that of wild-type JNK3. Potential sites for modification within the various binding sites of the enzyme may thus be identified. This information provides an additional tool for determining the most efficient binding interactions, for example, increased hydrophobic interactions, between JNK3 and a chemical entity or compound.

The structure coordinates are also particularly useful to solve the structure of crystals of JNK3 or JNK3 homologues co-complexed with a variety of chemical

entities. This approach enables the determination of the optimal sites for interaction between chemical entities, including candidate JNK3 inhibitors and JNK3. For example, high resolution X-ray diffraction data collected from crystals exposed to different types of solvent allows the determination of where each type of solvent molecule resides. Small molecules that bind tightly to those sites can then be designed and synthesized and tested for their JNK3 inhibition activity.

All of the complexes referred to above may be studied using well-known X-ray diffraction techniques and may be refined versus 1.5-3 Å resolution X-ray data to an R value of about 0.20 or less using computer software, such as X-PLOR [Yale University, ©1992, distributed by Molecular Simulations, Inc.; see, e.g., Blundell & Johnson, supra; Meth. Enzymol., vol. 114 & 115, H. W. Wyckoff et al., eds., Academic Press (1985)]. This information may thus be used to optimize known JNK3 inhibitors, and more importantly, to design new JNK3 inhibitors.

In order that this invention be more fully understood, the following examples are set forth. These examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention in any way.

EXAMPLE 1

Expression and purification of JNK3

A BLAST search of the EST database using the published JNK3α1 cDNA [S. Gupta et al. (1996)] as a query identified an EST clone (#632588) that contained the entire coding sequence for human JNK3a1. Polymerase chain reactions (PCR) using *pfu* polymerase (Strategene) were used to introduce restriction sites into the cDNA for cloning into the pET-15B expression vector at the NcoI and BamHI sites for expression of the protein in *E.*

coli. Due to the poor solubility of the expressed full length protein (Met 1-Gln 422), an N-terminally truncated protein starting at Ser residue at position 40 (Ser 40), corresponding to Ser 2 of JNK 1 and JNK2 proteins [S. Gupta et al. (1996)], preceded by Met (initiation) and Gly residues, was produced. The Gly residue was added in order to introduce an NcoI site for cloning into the expression vector. Further, systematic C-terminal truncations were performed by PCR to identify a construct that give rise to diffraction-quality crystals. This construct, which was prepared by PCR using deoxyoligonucleotides 5' GCTCTAGAGCTCCATGGGCAGCAAAAGCAAAGTTGACAA 3' (forward primer with initiation codon underlined) and 5' TAGCGGATCCTCATTCTGAA TTCATTACTTCCTTGTA 3' (reverse primer with stop codon underlined) as primers and confirmed by DNA sequencing, encodes amino acid residues Ser40-Glu402 of JNK3a1, preceded by Met and Gly residues, was used for structural studies described in this paper. Control experiments indicated that the truncated JNK3 protein has an equivalent kinase activity towards myelin basic protein when activated with an upstream kinase MKK7 in vitro (unpublished results).

E.coli strain BL21 (DE3) (Novagen) transformed with the JNK3 expression construct was grown at 30°C in shaker flasks into log phase (OD600 ~ 0.8) in LB supplemented with 100 µg/ml carbenicillin. IPTG was then added to a final concentration of 0.8 mM and the cells were harvested 2 hours later by centrifugation.

E. coli cell paste containing the truncated JNK3 protein was resuspended in 10 volumes/g lysis buffer [50 mM HEPES, pH 7.2, 10% glycerol (v/v), 100 mM NaCl, 2 mM dithiothreitol (DTT), 0.1mM PMSF, 2 µg/ml Pepstatin, 1µg/ml each of E-64 and Leupeptin]. Cells were lysed on ice using a microfluidizer and centrifuged at 100,000 x *g* for 30 min at 4°C. The 100,000 x *g* supernatant was

diluted 5 fold with Buffer A [20 mM HEPES, pH 7.0, 10% glycerol (v/v), 2 mM DTT) and applied to an SP-Sepharose (Pharmacia) cation-exchange column at 4°C. The column was washed with 5 column volumes of Buffer A, followed by 5 column volumes of Buffer A containing 50 mM NaCl. Bound protein was eluted with a 7.5 column volume linear gradient of 50-300 mM NaCl, and the truncated JNK3 protein was eluted between 150-200 mM NaCl. Eluted JNK3 protein from the SP-Sepharose column was dialyzed at ~ 1 mg/ml against Buffer B [25 mM HEPES, pH 7.0, containing 5% glycerol (v/v), 50 mM NaCl, 10 mM DTT] overnight at 4°C and centrifuged at 3,000 x *g*. The supernatant was concentrated by ultrafiltration (Centriprep-30, Amicon) to 10 mg/ml, centrifuged at 16,000 x *g* and stored at -70°C.

Example 2

Crystallization of JNK3

Full length human JNK3α1 has an 39-residue extension in the N-terminus when compared to JNK1, JNK2 and other MAP kinases [Fig. 1 and S. Gupta et al. (1996)]. We expressed the conserved MAP kinase homologous region of JNK3 without the first 39 residues for crystallographic studies. Initial crystallization trials yielded only small crystals that diffracted to 8Å. Since residues at the C-terminus of Erk2 and p38 are disordered [F. Zhang et al., Nature, 367, pp. 704-11 (1994); K. P. Wilson et al., J. Biol. Chem., 271, pp. 27696-700 (1996)], we reasoned that C-terminal portions of JNK3 might also be flexible and interfere with the formation of a well-ordered crystal lattice. We therefore searched for an active truncated JNK3 by combining limited proteolysis and systematic truncation of the protein. This screening approach resulted in the growth of larger, well-ordered JNK3 crystals. These crystals are grown from the JNK3 protein lacking the N-terminal 39 and C-terminal 20 residues. The truncated enzyme (residues Ser40-Glu402) displays wild-type kinase

activity when activated by MKK7 *in vitro*. All crystallographic studies were carried out using this form of the enzyme.

Crystallization trials were performed by combining the hanging-drop vapor diffusion technique and a sparse matrix search, in the presence and absence of MgAMP-PNP. No crystals were obtained in the absence of MgAMP-PNP, while crystallization trials carried out in the presence of MgAMP-PNP yielded an orthorhombic crystal form at 20°C over a reservoir solution containing 18-20% (v/v) polyethylene glycol monomethyl ether (average M_r = 550), 10% (v/v) ethylene glycol, 20mM β-Mercaptoethanol and 100mM Hepes (pH 7.0). The crystallization droplet contained a mixture of 1μL of reservoir solution plus 1mL of a protein solution that had been preincubated for one hour with 1mM AMP-PNP and 2mM $MgCl_2$ on ice. The crystals belong to the orthorhombic space group $P2_12_12_1$ (a=51.50 Å, b=71.24 Å and c=107.60 Å) with one enzyme molecule per asymmetric unit. The solvent content of the crystal is 44%. Before data collection, crystals were equilibrated in their reservoir solution for 2-5 minutes before flash-frozen in nitrogen gas for X-ray data collection at -170°C.

Example 3

X-Ray data collection and structure determination

X-ray data were measured on an Raxis IIC image plate, with mirror-focused CuKa X-rays generated by a rotating anode source. The diffraction images were processed with the program DENZO and data scaled using SCALPACK [Z. Otwinowski, In "Data Collection and Processing", L. Sawyer, N. Isaacs and S.W. Bailey, eds., Warrington, U.K.: Science and Engineering Council/Daresbury Laboratory. pp. 55-62 (1993)]. The data processing statistics are summarized in Table 1.

The starting phases for JNK3 were obtained by molecular replacement using coordinates of phosphorylated ERK2 as search model in the program AMoRe [J. Nazaza, Acta Crystallogr., A50, pp. 157-63 (1994)]. The Erk2 atomic model was modified by truncating to Ala for those residues that are different from JNK3 and deleting those loops that have significant insertions or deletions between Erk2 and JNK3. This hybrid model successfully produced rotation and translation function solution for JNK3, which provided a starting model with an R-factor=50% for reflections between 10 and 4.0 Å resolution, and an R-free=51% based on 10% of X-ray data set aside at the start of the refinement. Refinement of the model using both conventional least-squares and simulated-annealing procedures was done with X-PLOR [A. T. Brunger, XPLOR, Version 3.1 Manual, Yale University Press, New Haven, CT (1992)] using 8-2.3 Å data. The electron density corresponding to AMP-PNP molecule was visible from the map calculated using the initial model phases, but AMP-PNP was not included in the model refinement until the R-factor dropped to 28% and R-free to 39%. The refined model, at 2.3 Å resolution (Table 1), includes 339 residues of JNK3, one AMP-PNP molecule, two Mg^{2+} and 183 water molecules. The electron density maps revealed several discrete regions of disorder, leading the omission of some amino acid residues from the final model. N-terminal residues 40-44 and C-terminal residue 401 and 402 are disordered. In addition, two central regions of the enzyme are disordered, including residues 212-216 and 374-378. Finally, side chain atoms for Tyr223 was not modeled beyond Cβ due to poor electron density. The present R-factor is 22.1% (R-free=27.4%). Anisotropic scaling and bulk solvent correction were applied at the final stage of refinement (ref). The peptide torsion angles for 337 out of 339 well defined residues fall within most favored or generally allowed

regions of the Ramachandran plot, as defined in the program PROCHECK [R. A. Laskowski et al., J. Appl. Crystallog., 26, pp. 283-91 (1993)].

Example 4

Overall Structure

The crystal structure includes unphosphorylated JNK3 (residues 45-211, 217-373, 379-400), adenylyl imidodiphosphate (AMP-PNP, an ATP analogue) and two Mg^{2+} ions. Electron density for residues 40-44, 212-216 and 401-402 is not seen, and these amino acids are presumed disordered. The MAP kinase homologous region of JNK3 (Phe48-Glu397) is 45% identical in amino acid sequence to Erk2 and 51% to p38, whose structures have been reported (F. Zhang et al. (1994); K. P. Wilson et al. (1996); Fig 1). As expected, the overall architecture of JNK3 is highly similar to that of Erk2 and p38. The N-terminal lobe (residues 45-149, and 379-400) of JNK3 contains mostly beta-strands, whereas the C-terminal lobe (residues 150-211, 217-374) is predominantly alpha-helical. A deep cleft between the two domains comprises the ATP binding site, where the glycine-rich sequence of the enzyme (GSGAQGIV) forms a well defined β strand-turn-β strand structure over the nucleotide. The MAP kinase insertion in the C-terminal domain is 12 residues longer in JNK3 than in Erk2 and p38, resulting the N-terminal extension of helix αH and an extra 3_{10} helix, denoted 3/10(2)L14 between αH and α3L14 (Fig. 2a). We refer to this 12-residue insertion as "the JNK insertion" since it is present in all c-Jun N-terminal kinases [S. Gupta et al., (1996)]

The relative orientation of the N- and C-terminal domains is different between the structure of JNK3 and Erk2 (Fig 2b). Superposition of the C-terminal domain of JNK3 onto the corresponding lobe of Erk2 revealed a rotation of the N-terminal domain of JNK3 by

about 2.5° towards the active site. Despite the rotation of the domains, the structure of the individual domains of JNK3 and Erk2 are similar. Independent superpositions of the N- and C-terminal domains of Erk2 onto JNK3, ignoring the phosphorylation lip region, the JNK insertion and the protein termini, yielded protein backbone root mean square (rms) deviation of 1.23 Å for the N-terminal domain (JNK3 residues 56-149 and 382-400) and 1.60 Å for the C-terminal domain (JNK3 residues 150-208, 226-315 and 329-369).

Electron density is not visible for two disordered regions within the core region of JNK3. One region is Arg212-Thr216 in the C-terminal domain (Fig. 2a). The corresponding residues in Erk2 form beta strand β9 that precedes the "phosphorylation lip" (see below). In the unphosphorylated JNK3 structure, the disordered β9 indicates the flexible structure of the phosphorylation lip. The second disordered region includes residues Gln374-Lys378. These amino acids may be coupled to the activation state of the enzyme, since the structure of phosphorylated Erk2 showed that this portion of L16 is rearranged to a 3_{10} helix upon phosphorylation [B. J. Canagarajah et al., Cell, 90, pp. 859-69 (1997)].

Phosphorylation lip

We refer to the region spanning residues Thr217-Thr226, part of linker L12, as the "phosphorylation lip" or "activation loop", since it contains the regulatory phosphorylation sites Thr221 and Tyr223 (Fig. 2a). The conserved residue between two phosphorylation sites in the Thr-X-Tyr tripeptide sequence of JNKs is Pro, while it is Glu and Gly in Erk2 and p38 respectively. Most of the JNK3 residues in the phosphorylation lip are well ordered. The phosphorylation lip is four residues shorter in JNK3 than in Erk2 and two residues longer than in p38. The differences in length of the phosphorylation lip and the

center residues in the tripeptide sequence Thr-X-Tyr result in variations in the position and conformation of the activation residues, Thr221 and Tyr223 and in the path of the activation loop. Superposition of the C-terminal domain of Erk2 onto JNK3 reveals a 2.5 Å shift in the Cα position of Thr226 relative to Thr188 in Erk2 (Fig. 2b). The conformation of Thr226 is also different when compared to Thr188 in Erk2. In JNK3, a pair of water molecules are hydrogen bonded to the main chain carbonyl and amide groups of Thr226, and mediate interactions with the side chain of Lys199. As a result, Thr226 adopts a different Φ, Ψ angle (Thr226 of JNK3: Φ=-88° and Ψ=114°; Thr188 of Erk2: Φ=-46° and Ψ=130°), which redirects the path of the phosphorylation lip. The N-terminal portion of the lip makes van der Waals contacts with the αC helix, which in turn contacts the glycine-rich flap covering the nucleotide. Taken together, the protein backbone of the phosphosylation lip is well ordered, and takes up a conformation completely distinct from the corresponding residues in Erk2 and p38.

As a result of the conformation of the lip, the regulatory phosphorylation sites in JNK3 are differently positioned compared to that in Erk2 and p38. Thr221 and Tyr223 are 16 Å away from the location of the corresponding residues in Erk2 and 12 Å in p38. Despite the different locations, the regulatory threonine residues in all three enzymes are solvent exposed. In contrast, the local environments of the tyrosine residues are different. The side chain of Tyr221 is exposed to solvent in the JNK3 structure, and is disordered. The corresponding tyrosine residue in p38 is also exposed to the solvent, but its side chain is well-ordered and interacts with the hydroxyl group of Thr221 of p38 through a water molecule [K. P. Wilson et al, (1996)]. In contrast, the side chain of the corresponding tyrosine in Erk2 is buried. The Erk2 residue Tyr185 forms a

hydrogen bond with the side chain of Arg146, and makes van der Waals contacts with nearby hydrophobic amino acid side chains [F. Zhang et al. (1994)]. The side chain conformation of Tyr185 in the unphosphorylated Erk2 structure suggests that the phosphorylation lip must be refolded before Tyr185 can become a substrate for the Erk2 upstream activating kinase. A similar movement may not be needed to phosphorylate JNK3 and p38, since both threonine and tyrosine are accessible to the solvent in the unphosphorylated forms of JNK3 and p38.

Peptide substrate binding channel

The peptide substrate binding sites in JNK3 may be mapped by its homology to c-AMP-dependent protein kinase (cAPK, Fig. 1). The structure of the ternary complex formed by cAPK, PKI inhibitor and MnAMP-PNP [D. Bossemeyer et al., EMBO J., 12, pp. 849-59 (1993); D. R. Knighton et al., Science, 253, pp. 414-20 (1991)] shows that the peptide binding channel lies mainly in the C-terminal domain, and the position of the P+1 binding site is formed by a loop (residues Leu198 to Leu205) contiguous with the phosphorylation lip and connecting to αL12 (Fig. 3). The C-terminal domain of JNK3 superimposes well with that of cAPK, and it allows one to identify the residues that may be important for JNK3 peptide substrate binding. In JNK3, the protein backbone of residues Arg227 to Arg230 follows a similar path to the corresponding residues in cAPK (residues Pro202 to Leu205), with the side chain of Arg230 filling the P+1 site in an unfavorable conformation for the binding of the substrate (Fig. 3). While a portion of the phosphorylation lip (corresponding to residues Leu198-Thr201 in cAPK) takes a path distinct from that of cAPK and occupies the positions of P+1 and P+2 sites of the peptide substrate (Fig. 3).

Structural comparison of JNK3 and the phosphorylated Erk2 suggest how phosphorylation at Thr221

and Tyr223 of JNK3 might play a role in the activation of JNK kinases. In the phosphorylated Erk2, phosphothreonine pThr183 interacts directly with three arginine residues, Arg68 in αC, Arg146 in the catalytic loop (C loop) and Arg170 from the phosphorylation lip, while phosphotyrosine pTyr185 is ligated by Arg189 and Arg192 [B. J. Canagarajah et al (1997)]. Assuming that Thr221 and Tyr223 are ligated similarly in the phosphorylated form of JNK3, they would have to move by approximately 15 Å upon phosphorylation to be in close proximity with their ligands, and a large conformational change of the phosphorylation lip would be required. As a consequence of phosphorylation at Thr221 and Tyr223, restructuring of the phosphorylation lip may help to unblock the peptide substrate binding channel. These phosphorylation-related conformational changes in the phosphorylation lip as well as the peptide substrate binding channel have been observed in the crystal structures of low activity and phosphorylated Erk2 [F. Zhang et al. (1994); B. J. Canagarajah et al. (1997)].

Active site

Crystals of the binary complex of JNK3/MgAMP-PNP obtained from crystallizations of JNK3 in the presense of AMP-PNP and Mg^{2+} have allowed us to obtain structral data for the nucleotide-bound form of JNK3. As revealed in the structure, AMP-PNP is bound in the deep cleft between the two lobes of JNK3 (Fig 2a). The binding mode of the nucleotide analog is similar to that found in the ternary complex formed by cAPK, MnAMP-PNP and the inhibitor peptide PKI(5-24) [D. Bossemeyer et al. (1993)] (Fig 4a), which is believed to represent the bioactive conformation for protein kinases. These findings differ from previous crystal soaking experiments with Erk2 [F. Zhang et al. (1994)], and permit a more

detailed description of the interactions between the JNK3 and nucleotide.

The catalytic core of protein kinases contains a nucleotide binding sequence Gly-X-Gly-X-X-Gly-X-X that is refered to as "the glycine-rich phosphate anchor loop" due to its structural feature and role in the nucleotide binding [D. R. Knighton et al., Science, 253, pp. 407-13 (1991)]. The glycine-rich loop is well defined in JNK3, and superimposes well with that of cAPK, with an rms deviation 0.54 Å for the protein main chain atoms from Ile70 to Ser79 (Fig.4). The glycine-rich sequence Gly71-Ser-Gly73-Ala-Gln-Gly76-Ile-Val78 forms a flap over the nucleotide, covering it almost completely. The adenine base of the nucleotide is deep in the back of the domain interface, with its amino group (N6) making a hydrogen bond to the backbone carbonyl of Glu147, and N1 to the backbone amide of Met149. Non-polar interactions are also found at both sides of the purine ring, including Ile70 and Val78 from the glycine-rich flap on one side and Val196 from β7 on the other. The ribose O2' and O3' hydroxyls form a hydrogen-bonding network to the side chain of Asn152 and the carbonyl group of Ser193. The triphosphate group is tightly connected via hydrogen bondings, involving directly or indirectly, most of the invariant amino acids of protein kinases. Hydrogen bonds to phosphate oxygen atoms are formed by main chain amides of Gln75 and side chains of Gln75 and Lys93. Two magnesium ions (M1 and M2) are observed in the JNK3-MgAMP-PNP complex. The side chain carbonyl group of Asn194 is in close contact with M1 metal ion, which in turn bridges the oxygens of the α and γ phosphoryl groups of AMP-PNP. Asp207 interacts through water molecules with M2, which is bound to the β and γ phosphoryl group oxygens, while in cAPK, the corresponding residue (Asp184) directly coordinates both M1 and M2. This significant difference appears to be due to the inactive conformation of the JNK3 enzyme. An important role in

metal chelation has been proposed for Asp184 in cAPK which requires direct interaction of the aspartic residue with the metal ion [D. R. Knighton et al., Science, 253, pp. 407-13 (1991); D. Bossemeyer et al. (1993)]. Asp207 is located at a loop called the "DFG loop" preceding the disordered β9 in unphosphorylated JNK3. The structure of JNK3 suggests that upon phosphorylation, the refolding the phosphorylation lip and domain rotation should bring Asp207 closer to the nucleotide to allow its direct interaction with the metal ion.

Structural comparison of JNK3 and cAPK reveals that the two domains of JNK3 are rotated apart relative to their orientation in the structure of cAPK (Fig 3). This twist results in the misalignment of the two halves of the catalytic site of JNK3. From the N-terminal domain, the putative catalytic Lys93—takes the similar position of its equivalent residues in cAPK and forms hydrogen bonds to the oxygen atoms of α and β phosphoryl groups. However, the catalytic loop (Arg188-Asn194) and the DFG loop (Asp207-Gly209) in the C-terminal domain are misaligned (Fig4b). The conserved Asp189 and Asp207, both are thought to be essential for protein kinase activity [C. S. Gibbs et al., J. Biol. Chem., 267, pp. 4806-10 (1992)], are located 3 Å further away from Lys93 in JNK3, compared to that of their corresponding residues in cAPK. These differences suggest that the "open" conformation of the domains in JNK3 may contribute to the low activity of the unphosphorylated enzyme.

Similarity of JNK3 to Other Enzymes

The overall fold of JNK3 reveals similarities to the known structures of cAMP-dependent protein kinase and other MAP kinases, Erk2 and p38. The unphosphorylated JNK3 assumes an "open" conformation, in which the N- and C-terminal domains are oriented so that some of the catalytic residues are misaligned. In

addition, the phosphorylation lip partially blocks the substrate peptide binding site. The combination of these regulatory mechanisms suggests that both global (domain closure to bring the catalytic residues in close proximity) and local (refolding of the lip to relieve steric constraints to substrate binding) conformational changes are required for JNK3 activation.

Crystallographic studies of Erk2, p38 and JNK3 have shown that the region of β9 and the phosphorylation lip has the most diverse and labile conformation in unphosphorylated MAP kinases. This region of ERK2 adopts a conformation stabilized by interactions between the phosphates and residues near the activation loop in the phosphorylated enzyme [B. J. Canagarajah et al. (1997)]. Using the phosphorylated Erk2 structure as a model for the active conformation of JNK3 shows that the two phosphorylation sites, Thr221 and Tyr223, may play similar roles in activating JNK3 as they do in Erk2. In JNK3, phosphorylation of Thr221 may promote domain closure by interacting with Arg107 and Arg188, while phosphorylaton of Tyr223 may promote new interaction of the phosphotyrosine with Arg227 and Arg230, which in turn constitute the proline-directed P+1 pocket.

The JNK3/MgAMP-PNP binary complex is the first kinase structure of the JNK subfamily of MAP kinases to be determined. In the region spanning residues Ser40 to Ala418, JNK3 shares 92% and 87% amino acid identity with JNK1 and JNK2 , respectively. Thus, the JNK3 structure provides detailed structural information which provides insight into the mechanism of regulation for this class of MAP kinases. The variant residues among JNK isoforms are clustered in two regions (Fig 1). One region is the C-terminal portion of αF and its following loop L13. Most of the variant residues in this region are solvent exposed in the JNK3 structure and appear to be involved in substrate binding when compared with cAPK (Fig 5), suggesting their role in substrate binding specificity.

This is consistent with results obtained from the study of JNK chimeras which shows that JNK specificity towards c-Jun is directed to this region [T. Kallunki et al., Genes Dev., 8, pp. 2996-3007 (1994)]. Previous binding studies of JNK isoforms and various substrates further support this hypothesis and have shown that JNK isoforms with higher homology in this region display similar binding selectivity towards substrates [S. Gupta et al., 1996]. The other region is the α3L14 and JNK insertion, which lies on the protein surface next to the peptide substrate binding channel identified in cAPK (Fig 5). The location of this region suggests that it might be an extended substrate binding site in JNK kinase, and the sequence in this region may be important for substrate binding specificity.

Figures 1a-5 further depict the structure of the JNK3/MgAMP-PNP complex. Thus, Fig 1a depicts the structure-based sequence alignment of JNK3 [S. Gupta et al., (1996)], ERK2 [T. G. Boulton et al., Cell, 65, pp. 663-75 (1991)], p38 [J. C. Lee et al., Nature, 372, pp. 739-46 (1994)] and cAPK [M. D. Uhler, Proc. Natl. Acad. Sci. USA, 83, pp. 1300-04 (1986)]. The amino acid sequences of human JNK3, human ERK2, human p38 kinase, and murine cAPK are aligned based on structural similarity. The divergent N- and C-terminal regions of Erk2, p38 and cAPK are not shown. N- and C-terminal residues that are not included in the truncated JNK3 (JNK3: residues Ser40-Glu402) for crystallographic studies are denoted by lowercase letters. Residues in italic are not included in the model. Subdomains are labelled by Roman numerals according to S. K. Hanks et al., Science, 241, pp. 42-52 (1988). The secondary structural elements for JNK3 are indicated above the sequences (nomenclature as for Fig 2a), with open boxes designating αα helices and 3/10 helices and open arrows for ββ strands. Disordered regions are indicated with

dashed lines. Both JNK3 and cAPK sequence numbering are shown. Phosphorylation sites in the phosphorylation lip are denoted by an asterisk. JNK3 residues that differ from JNK1 and JNK2 are highlighted in bold.

Fig 2a is a ribbon representation of the overall fold of JNK3 complexed with MgAMP-PNP. Blue indicates secondary structural elements and loops conserved among protein kinases. Magenta indicates extensions and insertion characteristic of MAP kinases. The JNK insertion and the phosphorylation lip are colored cyan and red, respectively. The disordered region (residues 212-216) is indicated with dotted lines. Bound AMP-PNP and two Mg^{2+} ions are represented by space-filling models. The Cα positions of the regulatory phosphorylation sites Thr221 and Tyr223 are shown and labeled. Secondary structural elements are labeled according to ref. This diagram was constructed using RIBBONS [M. Carson, J. Appl. Cryst., 24, pp. 958-61 (1987)]

Figure 2b is a stereoscopic view of the superimposed structures of JNK3/MgAMP-PNP and Erk2 Cα representations of the structures of JNK3 (yellow and red) and Erk2 (blue and white) are shown after superposition of their C-terminal domains. Segments with largest structural divergence are labeled and highlighted in red and white, respectively.

Fig 3 is stereoscopic view of the superimposed structures of JNK3 and cAPK. Cα representation of JNK3 (yellow and red) and cAPK ternary complex (blue, white and green) are shown after superposition of their C-terminal domains. The phosphorylation lip is colored red in JNK3 and white in cAPK, and the PKI inhibitor is colored red, showing the difference in the conformation of the lip between two enzymes, and the lip of JNK3 occupying part of the peptide binding channel. MnAMP-PNP in cAPK ternary complex is omitted from the drawing and only the kinase catalytic core portion of cAPK is shown.

Figure 4a is stereoscopic view of the active

site of JNK3. Molecules of AMP-PNP and Mg2+ are shown together with their surrounding JNK3 residues. The AMP-PNP molecule is shown as thick bonds, and the protein residues as thin bonds. Two Mg^{2+} ions (colored orange and labeled M1 and M2) and two water molecules (colored cyan and labeled W1 and W2) are shown as spheres. Hydrogen bonds are indicated by dashed lines.

Fig 4b is a detailed comparison of the active site of JNK3 with that of cAPK. Cα representation of the ATP binding sites of JNK3 (yellow) and cAPK (blue) with the side chains of selected residues included. The atoms of AMP-PNP in the JNK3 binary complex and cAPK ternary complex have been superimposed. The N-terminal domains of the two enzymes are well aligned, while the difference in domain orientation results in the misalignment of the catalytic residues clustered in C loop and DFG loop, such as Asp189 and Asp207, with those in the N-terminal domain, such as Lys93.

Fig 5 is a substrate binding specificity of JNK isoforms. The solvent accessible surface of JNK3 is shown with the PKI inhibitor (drawn as orange tube) after the same superposition of JNK3 and cAPK structures done in Fig 3. Surface area corresponding to the JNK3 residues not conserved in JNK1 and JNK2 are colored red. Two clusters of divergent regions of JNK isoforms identified from the amino acid seqence alignment are located next to each other on the protein surface in the C-terminal lobe. The area containing αF and L13 has been shown to direct the substrate binding specificity toward cJun.

Example 5

The Use of JNK3/MgAMP-PNP Coordinates for Inhibitor Design

The coordinates of Figure 1 are used to design compounds, including inhibitory compounds, that associate with JNK3 or homologues of JNK3. This process may be aided by using a computer comprising a machine-readable data storage medium encoded with a set of machine-executable instructions, wherein the recorded instructions are capable of displaying a three-dimensional representation of the JNK3/MGAMP-PNP complex or a portion thereof. The graphical representation is used according to the methods described herein to design compounds. Such compounds associate with the JNK3 at the active site.

Example 6

JNK3 Activity Inhibition Assay

A. JNK3 activation

Five mg of JNK3 was diluted to 0.5 mg/ml in 50 mM HEPES buffer, pH 7.5, containing 100 mM NaCl, 5 mM DTT, 20 mM $MgCl_2$, 1 mM ATP. GST-MKK7(DD) kinase (the upstream mutant form of one of the activating kinases of JNK3) was added at a molar ratio of 1 GST-MKK7:2.5 JNK3. After 30 min at 25°C the reaction mixture was concentrated 5-fold by ultrafiltration in a Centriprep-30 (Amicon, Beverly, MA), then diluted back up to 10 ml and an additional 1 mM ATP added. This procedure was repeated three times to remove ADP and replenish ATP. The final (third) addition of ATP was 5 mM and the mixture incubated overnight at 4°C.

The activated JNK3/GST-MKK7(DD) reaction mixture was exchanged into 50 mM HEPES buffer, pH 7.5, containing 5 mM DTT and 5% glycerol (w/v) by dialysis or ultrafiltration. The reaction mixture was adjusted to 1.1 M potassium phosphate, pH 7.5, and purified by

hydrophobic interactions chromatography (at 25°C) using a Rainin Hydropore column. GST-MKK7 and unactivated JNK3 do not bind under these conditions and when a 1.1 to 0.05M potassium phosphate gradient is developed over 60 min at a flow rate of 1 ml/min, doubly phosphorylated JNK3 is separated from singly phosphorylated JNK.

Activated JNK3 (i.e. doubly phosphorylated) was stored at -70°C at 0.25-1 mg/ml.

B. JNK3 Inhibition Assay

To determine IC_{50} of the compound binding to JNK3, the kinase activity of JNK3 was monitored by coupled enzyme assay. In this assay, for every molecule of ADP generated by the JNK3 kinase activity one molecule of NADH is converted to NAD which can be conveniently monitored as an absorbance decrease at 340 nm. The following are the final concentrations of various reagents used in the assay: 100 mM HEPES buffer, pH 7.6, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 30 μM ATP, 2 mM phosphoenolpyruvate, 2 μM pyruvate kinase, 2 μM lactate dehydrogenase, 200 μM NADH, 200 μM EGF receptor peptide KRELVEPLTPSGEAPNQALLR, and 10 nM activated JNK3. First, all of the above reagents with the exception of ATP were mixed and 175 μl aliquots were placed per well of 96-well plate. A 5 μl DMSO solution of the compound was added to each well, mixed, and allowed to stand at 30°C for 10 minutes. Typically about 10 different concentrations of the compound were tested. The reactions were initiated with the addition of 20 μl of ATP solution. Absorbance change at 340 nm were monitored as a function of time. IC_{50} is obtained by fitting the rates vs. compound concentration data to a simple competitive inhibition model.

While we have described a number of embodiments of this invention, it is apparent that our basic constructions may be altered to provide other embodiments

which utilize the products, processes and methods of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the appended claims, rather than by the specific embodiments which have been presented by way of example.

Table 1. Summary of data collection and structure refinement

Data Statistics	Resolution (Å)	Reflections (Measured/Unique)	Completeness (%) (Overall/Outer Shell)	R_{merge} [1] (%) (Overall/Outer Shell)
	50-2.3	66063/16394	90.0/75.4	5.2/16.5

Structure Refinement

	Resolution (Å)	Number of Reflections	R-factor	Free R-factor	No. of water molecules	No. of AMP-PNP molecule	No. of Mg^{2+}
Data with F>2.0σF	30-2.3	14511	0.221	0.274[2]	18.3	1	2
Rms deviations	Bonds lengths 0.009Å		Bond angles 1.5°				

[1] $R_{merge}=100x\Sigma_h\Sigma_i| I_{hi} - \langle I_h \rangle | \ / \ \Sigma_h\Sigma_i| I_{hi}$.

[2] Free R-factor (ref, Brunger) was calculated with 10% of the data.